Biochemical Systematics and Ecology 51 (2013) 189–194

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Association of loganin contents with the genetic characterization of natural populations of *Palicourea rigida* Kunth determined by AFLP molecular markers

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ARTICLE INFO

Article history:
Received 4 June 2013
Accepted 24 August 2013
Available online 24 September 2013

Keywords:
Genetic diversity
Chemical variability
Cerrado
Rubiaceae

ABSTRACT

Extracts of the medicinal plant *Palicourea rigida* Kunth, popularly known as *douradinha*, are widely used for treating urinary tract disorders. Unfortunately, nowadays this is one of the species endemic to Brazilian Cerrado that is at greatest risk of extinction.

The aim of the this work was to use AFLP molecular markers to determine the genetic structure and diversity of eight natural populations of *P. rigida* and to associate their genetic characteristics with loganin production in order to obtain provide relevant information to promote programs for the conservation of this valuable medicinal plant.

A total of 120 polymorphic bands were scored and higher proportion of genetic diversity was found in inter-populations (64%) rather than in intra-populations (36%). F_{st} value was found to be significantly greater than zero (0.3601), demonstrating the complex genetic structure of *P. rigida* populations. Accessions collected from Cristalina, GO, showed higher percentage of polymorphic loci (65.5%) and the highest genetic diversity. Analysis of Molecular variance (AMOVA) demonstrated 63.9% of intra-population genetic variation. The lowest genetic variability was detected among accessions from the population found in Sacramento, MG. No spatial standard was observed for *P. rigida* population, suggesting a partially isolated island model. It was observed a minor but significant positive correlation ($r = 0.22$) between chemical and genetic matrices. The association between chemical and genetic data indicated that environmental factors promoted the loganin production in populations growing in Luziânia, GO, and therefore accessions from those populations should be considered as prime material for initiating the conservation process of *P. rigida*.

1. Introduction

Palicoura rigida Kunth is one of the species endemic to Brazilian Cerrado that is at greatest risk of extinction. According to Siqueira (2004), ecological data show evidence of the susceptibility of such species to temperature changes, indicating that they probably will be extinguished if the temperature of the planet increases by 1.8–2 °C. Moreover, because of its widely reported efficacy in the treatment of renal affections, this plant has been indiscriminately collected by inhabitants from the Cerrado biome (Rodrigues and Carvalho, 2001), thus compromising its survival. The medicinal effectiveness of *P. rigida* leaf extracts has been

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http://dx.doi.org/10.1016/j.bse.2013.08.032

attributed to loganin, an iridoid with analgesic and anti-inflammatory activity that is produced by the plant tissues (Xu et al., 2006; Park et al., 2010). Morel et al. (2011) have recently described that natural populations of that species produce variable concentrations of loganin.

Considering the above-mentioned findings obtained in this work, AFLP molecular markers were used to determine the genetic structure and diversity of eight natural populations of *P. rigida*. Additionally, the genetic characteristics of the plants were associated to their loganin production in order to provide relevant information to promote programs for the conservation of this valuable medicinal plant.

2. Materials and methods

2.1. Plant material

Leaves of *P. rigida* were collected during the month of March 2009 from eight natural populations located in Altinópolis and Rifaina (SP), Perdizes, Sacramento and Uberlândia (MG), Catalão, Cristalina and Luiziânia (GO). The collection sites were geo-referenced by using a Global Positioning System (Table 1). The plant material was stored in the Herbarium of Medicinal Plants at the University of Ribeirão Preto – UNAERP (voucher HPMU – 1416).

2.2. DNA extraction and AFLP analysis

Leaf tissues were used for DNA isolation by using the CTAB method (Doyle and Doyle, 1990). Quantity was estimated by spectrophotometry and quality was evaluated by using 0.8%-agarose gel electrophoresis in 1× TBE buffer.

Amplified fragment length analysis (AFLsP) was carried out according to protocol proposed by Vos et al. (1995) for genetic diversity determination. Restriction enzymes *Eco*RI and *Mse* I (New England Biolabs, Ipswich, MA, USA) were used for double digestion of 200 ng of genomic DNA. The resulting fragments were ligated to specific adapter sequences to complement the ends cleaved by both enzymes. Ligation reaction was kept at 37 °C for 3 h by using MJ RESEARCH PTC-100 thermal cycler.

After restriction and ligation reactions, the DNA was pre-amplified with primers containing one selective base, i.e., EcoRI-A/MseI-A. The PCR products from pre-amplification were used as template for the second reaction – the selective amplification. Ten combinations of 2 or 3 selective nucleotides were site-specifically attached to the 3′-terminal of the primers used.

Pre-amplification reaction used the following: 1 μl EcoRI + (1 oligo) (25 ng/μl); 1 μl MseI + (1 oligo) (25 ng/μl); 0.8 μl dNTPs 2.5 mM, 2 μl 10× Buffer B (Promega), 1.2 μl $MgCl_2$ (25 mM), 0.6 μl Taq DNA polymerase (5 un/μl) (Promega) and 2 μl of ligated DNA.

Amplification was carried out as follows: 1) 94 °C for 2 min, 2) 94 °C for 1 min, 3) 56 °C for 1 min, 4) 72 °C for 1 min, 5) 72 °C for 5 min; steps 2 to 4 were repeated 26 times. Amplification products were diluted 4 times and stored at 20 °C.

AFLP-PCR was performed as follows: 1 μl EcoRI + (3 oligo) (25 ng/μl); 1.2 μl MseI + (3 oligo) (25 ng/μl); 0.4 μl dNTPs 2.5 mM; 2 μl 10× Buffer B (Promega); 1.2 μl $MgCl_2$ (25 mM), 0.2 μl Taq DNA polymerase (5 un/μl) (Promega) and 1.5 μl pre-amplified DNA.

Selective amplification was carried out as follows: 1) 94 °C for 2 min; 2) 94 °C for 30 s; 3) 65 °C for 30 s; 4) 72 °C for 1 min; 5) 94 °C for 30 s; 6) 56 °C for 30 s; 7) 72 °C for 1 min; 8) 72 °C for 2 min, steps 2 to 4 were repeated 12 times and steps 5 to 7 repeated 23 times. Amplification products were stored at 20 °C.

After selective amplification, the product of each reaction was mixed with 8 μl of loading buffer. Next, the samples were denatured at 95 °C for 5 min and immediately transferred to ice.

AFLP products were separated in denaturing polyacrylamide gel (6%) with TBE 1× buffer at constant voltage of 80 W and maximum temperature of 50 °C during 4 h. The gel was stained with $AgNO_3$ and visualized with Na_2CO_3 according to protocol described by Creste et al. (2001).

2.3. Extraction and quantification of loganin

To confirm identification of the loganin sample, HPLC analyses were performed by using standard loganin (Merck).

Leaves (100 mg) collected from each population were separately ground (40 mesh), and the powdered material was extracted by using ultrasound-assisted extraction with 3 mL MeOH:H_2O (v/v) for 45 min. An aliquot of 1 mL was filtered and

Table 1
Places of collection of *P. rigida* – codes and geographic coordinates.

Mean values	Code	*n*	Latitude	Longitude	Altitude (m)
Rifaina (SP)	R	20	−20° 10′	−47° 28′	954
Uberlândia (MG)	UB	25	−19° 15′	−47° 43′	985
Luziânia (GO)	LU	20	−16° 21′	−47° 50′	947
Catalão (GO)	CA	20	−17° 49′	−47° 46′	940
Altinópolis (SP)	ALT	21	−21° 03′	−47° 29′	626
Sacramento (MG)	SC	21	−20° 01′	−47° 24′	647
Cristalina (GO)	CR	21	−16° 59′	−47° 14′	922
Perdizes (MG)	P	20	−19° 31′	−47° 05′	942

n = number of accessions collected.

analyzed with HPLC, with gradient mobile phase of (MeOH:(H_2O + CH_3COOH 0.1%) 10:90 (0 min) to (MeOH:(H_2O + CH_3COOH 0.1%) 50:50 (50 min) (Tian et al., 2007; Quian et al., 2007).

2.4. Statistical analysis

Binary data obtained from AFLP were used to estimate allele frequencies and were submitted to analysis of molecular variance (AMOVA) for decomposition of the total genetic variance between and within the populations, as proposed by Excoffier et al. (1992). Nei's diversity index (1978) was used in UPGMA clustering analysis (Ferreira and Grattapaglia, 1998) to demonstrate the genetic correlation between the populations investigated. The software packages GENES (Cruz, 2006), POPGENE (Yeh et al., 1999) and TFPGA (Miller, 1997) were used to carry out the analysis.

Chemical analyses were performed in triplicate and the experimental design was completely randomized.

Data were submitted to analysis of variance (ANOVA) by using the software SISVAR (Variance Analysis System, Federal University of Viçosa, MG, Brazil) (Ferreira, 2003), with means being compared by using Scott–Knott's multiple-range tests (Scott and Knott, 1974) at $P \leq 0.05$. This analysis was performed to cluster individual samples into groups depending on the loganin contents found in accessions collected in Luziânia, GO. Nei's genetic index (1978) was used to calculate the genetic distance among individuals and a dendrogram was created from a binary matrix by using the software POPGENE (Yeh et al., 1999).

The genetic similarity among the accessions collected in Luziânia were calculated with the Jaccard coefficient by using the NTSYS-pc version 2.01 software package (Rohlf, 2000). Both analyses used the Un-weighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method. The support values for the degree of confidence at the nodes of the dendrogram were analyzed by using the bootstrap re-sampling technique 1000 times. Matrices were correlated with Mantel statistics *Z* test by using the software GENES (Cruz, 2006).

3. Results and discussion

A total of 120 polymorphic bands of variable number of base pairs (75–350) were produced by 3 combinations of primers. The minimum and maximum numbers of polymorphic bands *per* primer were 20 and 59, respectively (Table 2).

The genetic characterization of the eight populations, determined by allele frequencies, is shown in Table 3. Among the 169 accessions investigated, the mean number of allele was 1545 and the mean percentage of polymorphic loci reached 54.5%. The *P. rigida* population from Cristalina presented the highest percentage of polymorphism (65.5%), followed by those from Uberlândia (64.9%), Altinópolis (62.5%), Luiziânia (56.9%), Catalão (54.9%), Perdizes (45.7%), Rifâina (43.0%), and Sacramento (42.8%). Nei's genetic distances (*h*) ranged from 0.2678 to 0.1494 and Shannon's index (*I*) from 0.4882 to 0.2228, whereas the population from Sacramento presented the lower diversity in both analyzed indexes.

Analysis of Molecular variance (AMOVA) demonstrated 63.9% of intra-population genetic variation.

The F_{ST} value of 0.3601 ($p < 0.001$), as stated by Sole-Cava (2001), revealed that this species has a complex genetic structure (Table 4). According to Frankham et al. (2007), F_{ST} value greater than 0.15 is usually an indicative of population fragmentation, that is, the higher this factor the lower the ability of dispersion, increasing the likelihood that these populations will become endogamous. The authors also reported that inbreeding and loss of genetic diversity are inevitable in small populations, reducing reproduction and survival rates in the short time. Moreover, endogamous populations have their capacity of evolving reduced in response to environmental changes in the long time, which increases the risk of extinction.

Geographically distant populations like those from Cristalina (GO) and Perdizes (MG) as well as those from Catalão (GO) and Altinópolis (SP), with geographic distances of 283 and 359 km and genetic distances ranging from 0.0528 to 0.0633, respectively, were grouped (Fig. 1 and Table 5).

No spatial pattern was observed because there was no significant correlation between genetic and geographic distances determined by the Mantel matrix correlation coefficient based on 1000 permutations. According to Diniz-Filho (2000), these results show that the genetic structure of *P. rigida* populations follows the partially isolated island model, evolving in the genetic space independently. A model of isolation by distance in which bordering populations would be genetically more similar was not evidenced.

The low gene flow ($Nm = 0.855$) among *P. rigida* populations resulted in a relatively high F_{ST} value (0.3601). This result suggests that the number of migrants *per* generation is inferior to 1, and that *P. rigida* populations are more susceptible to genetic drift.

Table 2
AFLP markers selected for molecular analysis of *P. rigida*.

Primer combinations	Total of bands	Number of polymorphic bands	Polymorphism (%)
E-AGA + M-TTC	50	20	60
E-AAT + M-TT	41	41	100
E-AG + TA	60	59	98
Total	151	120	79
Mean values	50	40	80

Table 3
Basic descriptive statistics for AFLP analysis of *P. rigida* populations.

Populations	$\overline{n}$	na	*h*	*I*	*P*
Rifaina (SP)	20	1.4305	0.1707	0.2485	43.05
Uberlândia (MG)	25	1.6490	0.2391	0.3530	64.90
Luziânia (GO)	21	1.5695	0.2107	0.3125	56.95
Catalão (GO)	20	1.5497	0.2145	0.3139	54.97
Altinópolis (SP)	21	1.6291	0.2521	0.3670	62.91
Sacramento (MG)	21	1.4238	0.1494	0.2228	42.38
Cristalina (GO)	21	1.6556	0.2678	0.2444	65.56
Perdizes (MG)	20	1.4570	0.1649	0.4882	45.70
Total	169	1.8609	0.3323	0.4882	–

Mean sample size (*n*), number of observed alleles *per* locus (na) Neis' índex (*h*); Shannon's index (*I*); percentage of polymorphic loci (*P*). POPGENE data (Yeh et al., 1999).

Table 4
Total genetic variance analysis (AMOVA) between and within populations of *P. rigida* based on binary data obtained from AFLP results.

Source of variation	D.F.	S. Q.	S. Q. M.	Variance components	% of total variation	*p*	F_{st} value
Between populations	7	1370.8093	195.8299	8.557	36.0071	<0.001	0.3601
Within populations	161	2448.3505	15.2071	15.2071	63.9929	–	–
Total	168	3819.1598	22.7331				

DF = degrees of freedom; SQ = sum of squares; SQM = sum of mean squares; *p* = level of significance for estimation of genetic variation based on 1000 permutations.

The results indicated that the AFLP technique was an efficient tool to determine the genetic diversity among the 8 populations of *P. rigida* investigated. Many works using AFLP molecular markers have equally validated the potential of this method for determining both diversity and genetic structure of plant populations, such as studies by Zhuravlev et al. (2008) with *Jatropha curcas*, Ribeiro et al. (2010) with *Maytenus ilicifolia* and Zang et al. (2010) with *Glycyrrhiza uralenseis*.

All accessions with high yields of loganin were from Luiziânia. Analysis of molecular variance clustered accessions of that population into 4 groups with significant difference in mean concentration of loganin. Group A had 5% of the accessions with high yields of loganin (205.14 mg/g PS); in Group B, 20% of the accessions contained medium yields of loganin (161.27 mg/g PS); in Group C, 55% of the accessions also presented medium contents (96.04 mg/g PS); and 20% of the accessions in Group D contained low yields of loganin (45.19 mg/g PS).

In the Luiziânia population, within a distance of 10 m, accessions producing either high, medium or low contents of loganin were collected. It was observed a minor but significant positive correlation ($r = 0.22$) between chemical and genetic matrices. The dendrogram presented in Fig. 2 shows a tendency to cluster accessions with medium contents of loganin, whereas the dendrogram in Fig. 3 shows that Group A is genetically more distant (0.2957) from the other two groups, with Groups B and C being more closely related (0.0410).

According to Dixon et al. (2006), natural products are synthesized by long and complex biosynthesis pathways that are controlled by a network of genes. The translation of genotype into chemotype is dependent on gene interactions and environmental factors. There are several studies describing the chemical profile of medicinal plants and others reporting their genetic data, but only a few works associate chemical and genetic information. Hu et al. (2007), established a variation pattern

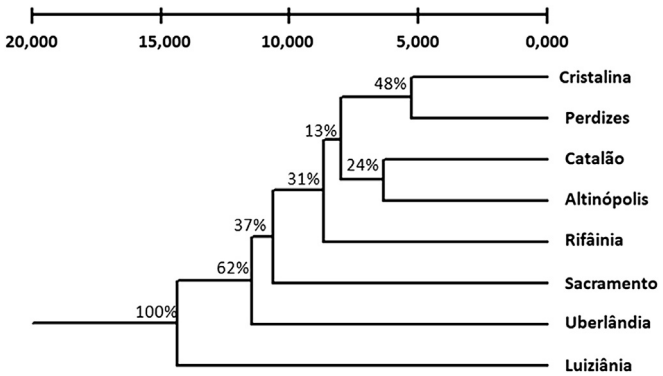

Fig. 1. UPGMA dendrogram based on Nei's (1978) genetic distance between *P. rigida* populations using TFPGA software.

Table 5
Genetic distance (Nei, 1978) below diagonal and geographic distance (Km) above diagonal.

	R	UB	LU	CA	ALT	SA	CR	*P*
R	***	104	425	262	94.09	18.97	354	79.90
UB	0.1162	***	324	160	200	90.37	258	72.52
LU	0.1918	0.1386	***	162	520	408	92.59	360
CA	0.0948	0.0935	0.1011	***	359	247	109	200
ALT	0.0710	0.0825	0.1209	0.0633	***	112	453	176
SA	0.0906	0.1277	0.1805	0.1142	0.1030	***	336	67.19
CR	0.0890	0.1123	0.1049	0.0546	0.0593	0.1080	***	283
P	0.0922	0.1548	0.1654	0.0908	0.1145	0.1171	0.0528	***

Data obtained using the software POPGENE and TRACKMAKER.

between the chemical and genetic diversities of *Vitex rotundifolia*, whereas Zang et al. (2012) investigated the association of chemical and genetic diversities of *Centella asiatica*, reporting no such a correlation and attributing the diversity among populations to environmental factors.

However, the present study has shown evidence of a correlation between chemical and genetic data and that environmental factors select genes that affect the loganin production of accessions from Luziânia population. However, the present study has shown evidence of a correlation between chemical and genetic data and that environmental factors select genes that affect the loganin production of accessions from Luziânia population. Lerin et al. (2007) compared genetic variability and chemical diversity of essential oils in *Salvia* spp. and they found a cluster tendency indicating a strong influence of genetic basis on the chemical composition, attributing this result to adaptations to edaphoclimatic conditions favoring some alleles at the expense of others.

Our data show that a conservation program through a germplasm bank should be undertaken with the introduction of greater number of individuals from the Luziania population in relation to the other populations studied.

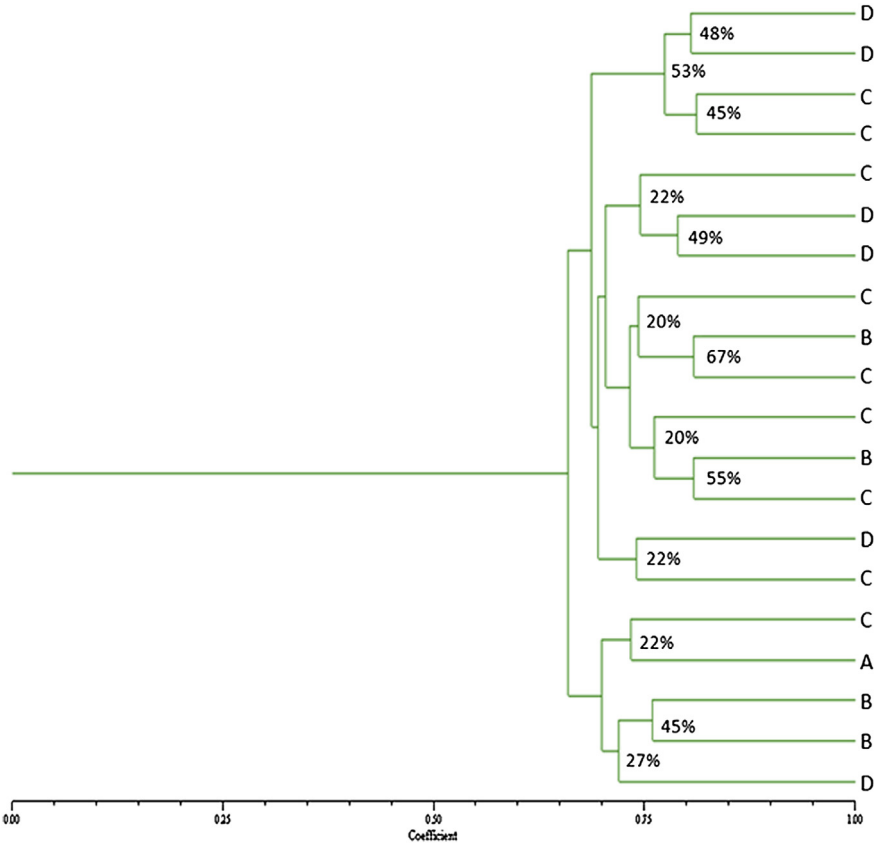

Fig. 2. UPGMA dendrogram based on AFLP data, showing the genetic relationships between *P. rigida* accessions from Luizianía using NTSYS software. Group A (high yields); B (medium yields); C (also presented medium contents); D (low yields) of loganin.

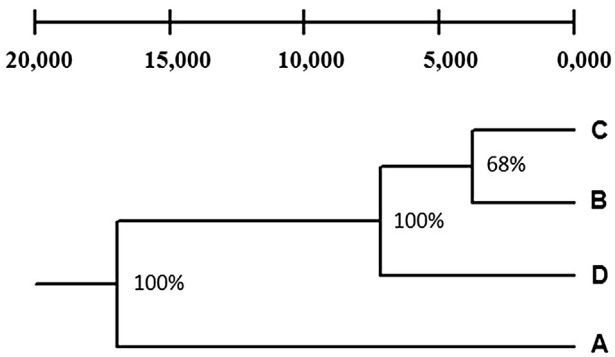

Fig. 3. UPGMA dendrogram based on Nei's (1978) genetic distance between the groups (A–D) using TFPGA software.

Acknowledgments

This research was supported by the State of São Paulo Research Foundation (FAPESP) under project number 07/58503-3.

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